Life Technologies™
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Explant & Seed Decontamination Kit

Product No. E2620

For use with *Phyto*Ready™ Media

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KIT COMPONENTS

Product No.	Product Description	Quantity
	Box	1
	Instruction Manual	1
B1438 – 1 ea	Spray Bottle	1
C1898	Culture Tube, Flip-Cap	2
P720 – 2 mL	Tween® 20	1
P067	Pipette, Plastic Transfer (1 mL)	2
W783 – 1L	Sterile Plant Tissue Culture Grade Water	1
B876 – 1 ea	Alcohol Burner	1
F081 – 1 ea	Forceps, Bayonet 7”	1
S963 – 1 ea	Scalpel Handle, No.3	1
S971	Scalpel Blades, No. 11	2

MATERIALS REQUIRED BUT NOT PROVIDED

1. Commercial chlorine bleach (6-8% sodium hypochlorite), identified as 100% in text
2. Liquid waste container to hold disinfection rinses
3. 70% isopropyl alcohol – for the spray bottle to disinfect surfaces
4. 95% ethanol – for the alcohol burner
5. *Phyto*Ready™ media or already gelled plant tissue culture media

SUGGESTED MATERIALS

1. Laminar flow hood with class 100 HEPA filtered air
2. Rubbermaid container if a laminar flow hood is not available
3. Autoclave/pressure cooker capable of maintaining 121°C (or 251°F) for 30 minutes
4. Nitrile gloves: Small (Product No. G941), Medium (Product No. G946), Large (Product No. G948), X-Large (Product No. G949)
5. Lint free towels/wipes (Kimwipes®)
6. Instrument Rest, Horizontal Bar (Product No. I4013)
7. Sterile surface to cut plant tissue such as autoclaved/pressure cooked paper towels in a suitable container or sterile petri dishes (Product No. D940)

INTRODUCTION

Developing effective explant & seed decontamination protocols for different species is usually a very large obstacle to overcome when first starting plant tissue culture. *Phyto*Technology Laboratories' Explant & Seed Decontamination Kit is designed to allow the user to disinfect vegetative tissue or seeds prior to introducing it to plant tissue culture media. Bacteria (e.g. *Bacillus* sp.), yeast, and fungi are ubiquitous in our environment (even indoor air) and plant tissue culture media are typically excellent growth media for these microorganisms.

This kit disinfects vegetative tissue or seeds from plants grown in soil with diluted sodium hypochlorite (Bleach) and subsequent water rinses so that explant tissue can grow (or seeds can germinate) on media. This kit provides the tools to sterilize instruments used to physically transfer disinfected tissue or seeds onto media. This kit was designed for use with our *Phyto*Ready™ media but it can be used for any gelled plant tissue culture media.

WORKING ASEPTICALLY

Aseptic is the adjective used to describe environments that are free from microorganisms (e.g. bacteria, yeast, fungi). Transferring explant tissue or seed is ideally performed under a laminar flow hood with class 100 HEPA filtered air. In the absence of working under a laminar flow hood, the user should find an area or a room with quiescent (very still) air flow. While working inside this room all windows, doors, and heating/air conditioning vents should be closed to prevent air disturbance. If more protection from contamination is desired beyond a room with quiescent air flow; an upside down Rubbermaid® tote with a rectangular 2.5' wide x 1.5' tall cutout (see Figure 1.)on one side can be used as a 'Still-Air Transfer Chamber' for an aseptic working environment (AWE).

Figure 1. An example of a 'Still-Air Transfer Chamber'

70% Isopropyl Alcohol (IPA) is the best multi-purpose surface disinfectant. Spraying this on a surface and then wiping with a lint-free towel/wipe and allowing the surface to fully dry is considered a good practice in aseptic technique. Prior to initiation of all work, all surfaces in an AWE should be sprayed with 70% IPA including all inner surfaces of a Rubbermaid® tote. All spills in an AWE should be wiped up immediately and re-sprayed with 70% IPA.

All materials (e.g. capped media tubes, instruments, etc.) should be sprayed with 70% IPA and prior to entering the aseptic working environment; this includes gloved hands. Once inside the AWE the materials should be allowed to dry. NOTE: Even after spraying gloved hands they should never be considered sterile and every time gloved hands exit the AWE, they need to be re-sprayed prior to re-entering.

If materials that come into contact with sterile material are not previously gamma-irradiated and can tolerate high heat and pressure, a good practice is to autoclave or pressure cook instruments (e.g. forceps, scalpels, etc.) prior to first use, and then flame immediately with the alcohol burner before using. Be sure to allow instruments to cool after flaming for a few minutes prior to touching plant tissue.

Explants or seeds shouldn't be sprayed with 70% IPA. Avoid introducing heavily contaminated material (soil, contaminated plant tissue culture containers) to the AWE. Plant material taken from soil/vermiculite mixtures should be rinsed with water prior to entering the AWE.

One key fundamental in aseptic technique is that you never want to allow any non-sterile object (e.g. gloved hands, non-flamed forceps, etc.) to block the laminar flow air in front of what would be considered a sterile object (e.g. uncapped tubes of prepared media, disinfected tissue or seeds, already established plant tissue cultures in media, etc.). If using a Rubbermaid® tote or working in a low-air flow room; do not pass anything that is non-sterile over the top of sterile objects.

Every time the user touches anything (e.g. disinfected tissue, a sterile surface, etc.) with the forceps or scalpel, they need to be re-flamed for a few seconds and allowed to cool before touching tissue or seeds again.

Minimize the amount of time the *Phyto*Ready™ media tubes or gelled plant tissue culture media are un-capped and exposed to the air in the AWE. The amount of time disinfected tissue is exposed to air in the AWE should also be minimized.

Always work with the cleanest material at the beginning of each work cycle as opposed to what might be considered the most unclean or suspect of contamination. For example material from a greenhouse would be considered cleaner than plant tissue grown outdoors.

If an object that is used to handle sterile materials in the AWE falls on the floor, it is best to completely re-sterilize the object with an autoclave/pressure cooker. Floors are generally considered to have the highest population of microorganisms in a room.

To see video of some guidelines for aspetic technique please see our 'Aseptic Transfer Techniques' on our YouTube Channel – PhytoTechnology Laboratories.

EXPLANT DISINFECTION

The goal of tissue disinfection is to expose your plant tissue to the least harsh conditions (e.g. incubation time, bleach concentration) that will still remove microorganisms. The following is merely a guideline, and your tissue may require more or less time in more or less concentrated bleach solutions. If your tissue is particularly sensitive to even dilute bleach solutions at short periods of time and contamination is still a problem, the user may want to increase the volume of dilute bleach solutions relative to the approximate mass or volume of the explant material, and decrease the time exposed. It is better when first performing a disinfection to start small, and determine a proper protocol for 1-2 pieces of tissue and later scale-up once it has been found.

1. Prepare a disinfection solution, or 100 mL of a 1:10 diluted commercial bleach solution (0.6-0.8% sodium hypochlorite solution) in a flip-cap tube (Product No. C1898).
    a. Add 10 mL (1/3 fl. oz.) of commercial bleach
    b. Add 90 mL (3 fl. oz.) of W783
    c. Add 4-5 drops of P720 with P067
    d. Cap the C1898, and invert 4-5 times or until the solution appears homogeneous
    e. NOTE: Sodium hypochlorite will damage the P720 after long periods of time, so prepare each solution fresh the day a disinfection protocol is performed
2. Spray the disinfection solution (10% bleach) flip-cap tube with 70% IPA and place in the aseptic work environment (AWE).
3. Obtain plant tissue:
    a. Some plant tissue protocols require rinsing the tissue off under running tap water for 5-30 minutes depending on the softness. Make sure where ever this is performed that the sink has been thoroughly cleaned.
    b. Transfer rinsed plant tissue to the AWE
4. Remove young juvenile tissue from plant and cut ½" to 1" lengths of tissue with the scalpel.
5. Add small amount of explant tissue to the 100 mL of disinfection solution (10% bleach) in C1898 and cap it.
6. Gently invert the C1898 flip-cap tube several times for 5-15 minutes.
7. After the tissue has been added and it is being incubated, this is a good time to clean your AWE with 70% IPA to remove any contaminants that may have been brought into the area with the rinsed plant tissue.
8. Decant the disinfection solution off into a waste container being careful not to pour out the tissue. NOTE: Sometimes it is helpful to pipette off the solution, but the pipette must be sterile. Single-use serological pipettes work well for this, such as product numbers P995 (25 mL) and P996 (50 mL), sold separately.
9. Add 100 mL of W783 to the tissue in the C1898 flip-cap tube, and invert several times for approximately 1-2 minutes.
10. Still under the AWE, decant the water off into a waste container being careful not to pour out the tissue.
11. Repeat steps 6-7 for 2-3 more times.
12. Depending on the type of tissue being disinfected, it is sometimes beneficial to trim off a few millimeters of tissue exposed to solution on a sterile petri-dish (Product No. D940) to prevent oxidized tissue from spreading.
13. Flame the forceps (Product No. F081) for 3-5 seconds in the alcohol burner and allow the instrument to cool on I4013 (optional) for a few minutes, or hold in hand for a few minutes.
14. Transfer the tissue with the forceps to a *Phyto*Ready™ media tube or other gelled media container.
15. Cap the media container & place ~20" below a fluorescent light with a timer set for 16 hrs day/8 hrs night. The temperature of where the material is grown is highly species dependent, but 25°C (or 77°F) is a common optimal temperature.

Bacterial contamination will generally appear in 2-3 days on the surface of the tissue if the explant decontamination is unsuccessful, and Fungi will typically appear in 7-10 days. This protocol is specific to the users explant tissue and the micro-organism population it was in, and this needs to be determined empirically if no prior literature exists. In heavily contaminated explant tissue or seeds, one of the more stringent protocols' used in plant tissue culture is for corn seed and is as follows:

1. Immerse tissue (few kernels) in 50 mL of 95% ethanol in a C1898 flip-cap tube for 0.5-2 minutes while gently inverting.
2. Decant off the ethanol solution off into a waste container under the AWE being careful not to pour out the tissue.
3. Prepare a disinfection solution, or 100 mL of a 1:2 diluted commercial bleach solution (3-

4% sodium hypochlorite solution) in a separate C1898 flip-cap tube.
   a. Add 50 mL (1&2/3 fl. oz.) of commercial bleach
   b. Add 50 mL (1&2/3 fl. oz.) of W783
   c. Add 4-5 drops of P720 with P067
   d. Cap the C1898, and invert 4-5 times or until the solution appears homogeneous
   e. NOTE: Sodium hypochlorite will damage the P720 after long periods of time, so prepare each solution fresh the day a disinfection protocol is performed
4. Add 50 mL of disinfection solution (50% bleach) to the container with corn kernels and cap it. NOTE: This is a very harsh disinfection procedure, and many plant species cannot survive this.
5. Gently invert the C1898 container several times for 15 minutes.
6. Decant off the disinfection solution (50% bleach) off into a waste container being careful not to pour out the tissue.
7. Add the remaining 50 mL of disinfection solution (50% bleach) to the container with corn kernels and cap it.
8. Gently invert the C1898 container several times for 15 minutes.
9. Decant off the remaining disinfection solution (50% bleach) off into a waste container being careful not to pour out the tissue.
10. Add 50 mL of W783 to the tissue in the C1898 container, and invert several times for approximately 1-2 minutes.
11. Decant the water off into a waste container being careful not to pour out the tissue.
12. Repeat steps 6-7 for 2-3 more times.
13. Place on the appropriate media aseptically.

Depending on the species and rate of growth, most plant tissue is sub-cultured onto new media every 30-60 days.

SEED DISINFECTION

Seed coats are known to contain crevasses that can house micro-organisms. Like tissue disinfection, the goal of seed disinfection is to find the least concentrated bleach or disinfectant solution and least amount of time in that solution that will remove microorganisms, yet still allow for germination. Some seeds must undergo a dormancy period where they need to be exposed to cold temperatures 2-8°C (or 36-46°F) for weeks, or be kept in the dark for many days, or both. It is important that dormancy period has been overcome prior to initiating the seed disinfection because any protocol performed where dormancy was not overcome could provide a false negative (i.e., the combination of bleach concentration with time disinfected was lethal to the seed). It should also be noted that some seeds have low germination rates, and the proper amount of seed should be used to increase the chances of germination. To provide an example, if you are germinating a seed which is known to only have a germination rate of 10%, working with only one seed has a 1 in 10 chance of success. Whereas starting with 20 seeds would double the chances of obtaining germinated seed.

As discussed in the 'Explant Disinfection' section, this is merely a guideline and your seed may require more or less time in more or less concentrated bleach solutions. Please see the Orchid Seed Germination section of our Orchid Media Selection Guide (in the 'Technical Info' section of our website) for more information.

1. Prepare a disinfection solution, or 50 mL of a 1:10 diluted commercial bleach solution (0.6-0.8% sodium hypochlorite solution) in a C1898 flip-cap tube.
   a. Add 5 mL (1/6 fl. oz.) of commercial bleach

   b. Add 45 mL (1.5 fl. oz.) of W783
   c. Add 2-3 drops of P720 with P067
   d. Cap the C1898, and invert 4-5 times or until the solution appears homogeneous
   e. NOTE: Sodium hypochlorite will damage the P720 after long periods of time, so prepare each solution fresh the day a disinfection protocol is performed
2. Enter the disinfection solution (10% bleach) into the aseptic work environment (AWE)
3. Add approximately a 1-5 mL volume of seeds to the 50 mL of disinfection solution (10% bleach) in C1898 and cap it.
4. Gently invert the C1898 container several times for 5-15 minutes.
5. Decant the disinfection solution off into a waste container being careful not to pour out the seeds. NOTE: Sometimes it is helpful to pipette off the solution, but the pipette must be sterile. Single-use serological pipettes work well for this, such as product numbers P995 (25 mL) and P996 (50 mL), sold separately.
6. Add 50 mL of W783 to the tissue in the C1898, and invert several times for approximately 1-2 minutes.
7. Decant the water off into a waste container being careful not to pour out the seeds
8. Repeat steps 6-7 for 2-3 more times.
9. Flame the forceps for 3-5 seconds in the alcohol burner and allow the instrument to cool on I4013 (optional) for a few minutes, or hold in hand for a few minutes.
10. Transfer the seeds with the forceps to a *Phyto*Ready™ media tube or other gelled media container.
11. Place the container in an appropriate light environment which encourages germination. The temperature of where the material is grown is highly species dependent, but 25°C (or 77°F) is a common optimal temperature.

Germination can occur for many herbaceous species in 3-14 days, but for orchids it can take many months, and conifers can take a year or more without breaking dormancy. It should be noted that some seeds after breaking cold dormancy may prefer darkness once disinfected and placed on media. It is recommended that the user consult the literature.

TROUBLESHOOTING

The decontamination was successful, but the tissue has browned in color significantly (a sign of overstilization).

1. Soak the tissue in an autoclaved antioxidant mixture (Product No. A126) dissolved solution for 5-15 minutes (can be much longer, just a guideline) following the final water rinse. NOTE: This solution can also be added to media.
2. Cut-back on ends of tissue exposed to disinfection solution following the rinses. NOTE: At this point, it is important to cut the tissue on a sterile surface such as petri-dishes (Product No. D940) or autoclaved paper towels handled aseptically.

Tissue is growing or seeds have germinated but contamination remains

1. Increase the amount of P720 added to the solution, if the amount of tissue surface area relative to the volume of disinfection solution is high.
2. Lower the pH of the disinfectant solution to pH 5.5, as this will increase the maximum amount possible of hypochlorous acid (active ingredient in bleach) present relative to chlorite ion. NOTE: Commercial bleach diluted solutions are often very alkaline pH >10.
3. Increase the bleach concentration and time tissue/seed is incubated in disinfection solution.
4. Perform back-to-back disinfectant solution incubations with fresh disinfection solution each time.

5. Add 95% ethanol to the tissue/seeds for a few minutes prior to adding bleach disinfection solution.

Concern over alkaline pH of bleach solution causing phytotoxicity

Sodium dichloroisocyanurate (Product No. D253) can liberate hypochlorous acid (HOCl) in water at neutral pH, and frequently does not need to be rinsed. NOTE: Sodium dichloroisocyanurate is typically used at 2-5 g/L, and often the incubation times needed are longer than when using bleach solutions .

If many attempts at explant/seed decontamination are unsuccessful

We offer a product called PTC[3] (Product No. P6820) which contains both bacteriostatic and fungistatic agents to inhibit the growth of microorganisms in plant tissue culture. This product can be added to media and autoclaved, or added to tissue during later stages of the disinfection process.

REFERENCES

Kyte L, Kleyn J, Scoggins H, and M. Bridgen (2013) Plants from Test Tubes: An Introduction to Micro-propagation. Timber Press. Portland, OR.

NOTES

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